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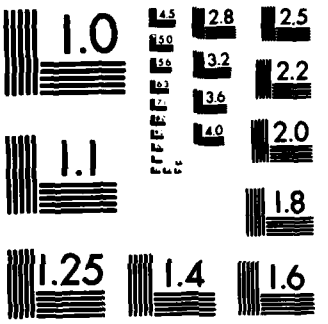


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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Two classes of synthetic immunoadjuvants, the muramyl di-peptides, and the polyribonucleotide complexes, have the capacity to either elevate or suppress the immune response depending on the time of their administration relative to antigen. The long term objectives of this contract are to define the cell and molecular signals associated with the in vivo suppression of the immune response of Balb mice by each of these classes of non-toxic adjuvants. During the first two years the following findings have been made:

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(1) Optimal conditions for suppression of anti-SRBC antibody by a single injection of each adjuvant have been determined. Non-specific suppression appears to last approximately 7 days.

(2) Both T cells and an adherent cell population have been found capable of transferring MDP induced suppression to syngeneic mice when administered with antigen.

(3) Preliminary experiments indicate a soluble factor mediates MDP induced suppression.

(4) Suppression induced by polyadenylic-polyuridylic acid complexes contrary to MDP could not be transferred via spleen cells to syngeneic mice when administered with antigen to the latter. However, suppression was exerted in recipient mice when such cells were transferred two days after antigen.

(5) Addition of poly A·poly U to the mixed leucocyte reaction as a model of cell mediated immunity over a dose range of 0.001-100 µg failed to suppress this reaction. However, serum removed 90 minutes after injection of poly A·poly U uniformly suppressed the MLR. This factor was found to have a molecular weight greater than 30,000 and to be stable at 56°C for 30 minutes. Assays for gamma interferon were negative; however, poly A·poly U sera contained alpha interferon and poly I:poly C suppressive sera had beta interferon.

## Suppression of the Antibody Response by MDP and Derivatives

A single injection of MDP (300 µg) ip or iv to BALB mice one day prior to antigen (SRBC) in repeated experiments induces an approximate 50% suppression of splenic PFC (cf Annual Report #1). To determine the duration of this non-specific suppression, MDP was injected from 1-13 days prior to injection of antigen and PFC's determined 4 days after antigen. The results are shown in Table I and illustrate that a single injection of MDP gives rise to a suppressive state lasting approximately one week. Of interest was the similar degree of suppression persisting throughout the 1-7 day period and its relative rapid drop off on day 10. The variation seen in response to these immunomodulating agents is commonplace, despite inbreeding. Confirming experiments are underway.

TABLE I

The Duration of Suppression Induced by MDP in vivo*

| MDP Injected I.P. on Day | Anti-SRBC PFC/2 x $10^5$ Spleen Cells | | Percent Suppression (Average) |
|---|---|---|---|
| | Expt. 1 | Expt. 2 | |
| -1 | 113 ± 4 (61)<br>265 ± 3 (9) | 9 ± 4 (98)<br>51 ± 3 (87) | 64 |
| -2 | | 0 ± 0 (100)<br>298 ± 5 (27) | 64 |
| -4 | | 203 ± 3 (49)<br>257 ± 13 (35) | 42 |
| -5 | 61 ± 5 (79)<br>308 ± 13 (0) | | 40 |
| -7 | 63 ± 7 (78)<br>297 ± 9 (0) | 20 ± 1 (95)<br>275 ± 15 (30) | 51 |
| -10 | 182 ± 2 (38)<br>345 ± 3 (0) | 495 ± 10 (0)<br>507 ± 10 (0) | 10 |
| -13 | 255 ± 3 (12)<br>283 ± 2 (3) | | 7 |
| Control (No MDP) | 270 ± 5<br>297 ± 3<br>306 ± 21 | 394 ± 14<br>395 ± 16 | |

*SRBC ($1 \times 10^8$) given ip on day 0. PFC assayed on day +4.

( ) = % suppression relating to control average.

In Table II is seen the results of attempts to transfer the suppression to non-x-irradiated syngeneic mice following more rigorous separation techniques than those recorded in our previous report. Both adherent and non-adherent cells transferred a statistically significant degree of suppression as was seen in multiple earlier experiments (cf Annual Report, #1). A striking change was seen once again when separated T and B cell populations were transferred individually. A major suppressive influence appeared to lie with the T cells, whereas the B cell population resulted in a marked enhancement of PFC's. Accordingly, the figures for whole as well as non-adherent spleen cells must represent an equilibrium between the positively responding B cell population and the negative impact exerted by suppressor T cells. Indeed, it suggests the responses observed in vivo are net effects, and that antigen also has stimulated the "help" necessary to result in fully functional B cells in the presence of suppressor cells but which cannot be expressed until the latter are quenched or in this case, removed.

TABLE II

Cellular Transfer in vivo of MDP-Induced Suppression

| Cells Transferred* ($1 \times 10^7$) | PFC/$2 \times 10^5$ cells following injection in vivo of PBS | MDP** | Per Cent Suppression |
|---|---|---|---|
| Whole Spleen | 265 ± 10 | 239 ± 10 | 10 (0.14) |
| Adherent | 176 ± 8 | 137 ± 5 | 22 (0.02) |
| Non-adherent | 104 ± 8 | 85 ± 3 | 19 (0.12) |
| B | 116 ± 6 | 193 ± 9 | ↑67 (0.004) |
| T | 153 ± 6 | 72 ± 4 | 53 (0.001) |
| | 126 ± 3 | 35 ± 2 | 72 (0.000) |

*Cell populations separated by adsorption to BHK cell exudate coated flasks, Sephadex G10 columns and panning.

**300 µg MDP injected ip on day -1. In vivo MDP positive controls (no transfers) resulted in 30% suppression. $1 \times 10^8$ SRBC injected ip on day 0 as were the transferred cells.
( ) = p value.

Inasmuch as we previously had been able to transfer MDP induced suppression in vivo consistently also with adherent spleen cells, we tested the capacity of thioglycollate induced peritoneal macrophages incubated with MDP for one day in vitro, to transfer suppression. The results are in Table III and show that such a procedure also resulted in diminution of PFC's and should prove useful as a model to define the suppression exerted by this cell. The phenotypic expression of membrane antigens by both suppressive macrophages and T cells is under study.

TABLE III

Transferrable Suppression Induced in Peritoneal Macrophages by MDP In Vitro

| Transfer of Macrophages on Day | PFC/2 x $10^5$ cells following transfer of macrophages pre-treated with | | Percent Suppression |
|---|---|---|---|
| | PBS | MDP* | |
| -2 | 248 ± 9 | 177 ± 3 | 29 (0.009) |
| -1 | 281 ± 3 | 147 ± 5 | 48 (0.0001) |

*MDP, 3 µg/ml, added to 4 x $10^6$ BALB macrophages, incubated 2 hours, washed and 5-6 x $10^6$ viable cells injected ip 1 or 2 days before SRBC, 1 x $10^8$ given ip. ( ) = p value.

To determine whether suppression was exerted on a T independent antigen as well as a T dependent antigen, the experimental data depicted in Table IV were acquired. It may be seen that MDP injected into mice 1 or 2 days before addition of TNP-Brucella abortus to culture dishes of spleen cells removed from such mice was not capable of suppressing the PFC response to this thymus independent antigen, while a very effective suppression of SRBC was exerted.

TABLE IV

Lack of Suppression by MDP of a T-independent Antigen

| Antigen (Day 0) | PFC/Culture | | Percent Suppression |
|---|---|---|---|
| | PBS | MDP* | |
| SRBC | 415 ± 16 | 14 ± 3 | 97 (0.001) |
| TNP-BA | 505 ± 45 | 470 ± 35 | 7 (0.6) |

*MDP, 300 µg given ip on day -1; SRBC, mice sacrificed on day 0, spleen cells placed in culture and 1 x $10^7$ SRBC/plate or 3 x $10^4$ TNP-Brucella abortus cells added. PFC assayed on day +4.

To determine whether mature thymus cells were required by MDP to induce full suppression, the effect of this adjuvant in nu/nu mice was studied. Thus, MDP was injected ip without antigen into euthymic and athymic groups of mice, spleens removed a day later and single cell suspensions ($5 \times 10^6$) added along with antigen (SRBC) to an equal number of normal, euthymic spleen cells in in vitro culture. Analysis of PFC's 4 days later revealed the lack of ability of MDP to generate suppression in athymic BALB mice (cf Table V). Consequently, this first experiment suggests that MDP may be unable to induce suppression in the macrophage population without T cells present. This is being tested further in current experiments.

TABLE V

Lack of Ability of MDP to Induce Suppression in Athymic Mice

| Mice | Spleen cells from MDP injected mice | Normal Spleen Cells | PFC/culture* | Percent Suppression |
|---|---|---|---|---|
| Euthymic | — | + | 940 ± 68 | |
| | + | — | 365 ± 57 | 61 |
| | + | + | 365 ± 22 | 61 |
| Athymic | — | + | 730 ± 39 | |
| | + | — | 20 ± 36 | |
| | + | + | 1005 ± 34 | 0 |

*All single or mixed cell cultures contained a total of $1 \times 10^7$ cells. PFC's assayed on day +4.

Experiments also have been initiated to determine whether MDP induced suppression is mediated by a secreted molecule. Accordingly, mice were injected ip with 300 µg MDP or medium, the spleens removed 24 hr later and 1 x $10^7$ whole spleen cells or purified T, and B+ macrophage populations cultured for either 6 or 48 hours. The supernatant fluids were collected and 0.2 ml added to 1 x $10^7$ normal spleen cells along with antigen. PFC's were assayed 4 days later. The results are shown in Table VI. A secreted suppressive factor is indicated in the T cell supernatant fluid collected at 48 hours and to a somewhat lesser extent in the macrophage + B cell population. On the other hand, supernatant fluids from cultured normal macrophages or T cells enhanced PFC formation.

TABLE VI

Suppression of the In Vitro PFC Response by Cultured Supernates from Spleens Exposed to MDP

| Cellular Source of Supernate | PFC/Culture following | | | |
|---|---|---|---|---|
| | 6 hr Sup | % Suppression | 48 hr Sup. | % Suppression |
| MDP whole spleen | 1585 ± 44 | 0 | 500 ± 14 | 28 |
| MDP MØ + B cells | 1135 ± 44 | 28 | 560 ± 37 | 19 |
| MDP T cells | 1390 ± 30 | 11 | 410 ± 22 | 41 |
| Control | 1565 ± 44 | | 690 ± 22 | |
| Normal whole spleen | 705 ± 54 | | 1025 ± 69 | |
| Normal MØ | 1150 ± 60 | | 1085 ± 48 | |
| Normal B cells | 420 ± 16 | | 380 ± 24 | |
| Normal T cells | 600 ± 35 | | 1100 ± 25 | |
| Control | 435 ± 36 | | 350 ± 8 | |

To determine whether the capacity of Concanavalin A to induce Interleukin-2 secretion from T cells was dampened by prior injection of MDP and thus explain the suppression, the following experiment was performed. BALB/c mice were injected with either 300 µg MDP or 0.5 ml PBS ip 24 hr before sacrifice. Single spleen cell suspensions were then incubated for 24 hrs with or without Con A and the supernatant fluids collected and tested for Il-2 activity in a co-stimulator assay. There was no statistical difference under these conditions in 3 experiments in the ability of Con A to release Il-2 from the MDP injected animals as compared to the controls (96% ± 37 vs 91% ± 44 respectively).

Many derivatives of MDP with different biological activities have been synthesized. Testing of two of these which are currently undergoing intensive scrutiny was initiated for their capacity to suppress antibody forming cells under our conditions. The first was N-acetyl-muramyl-L-alanyl-D-glutamine-n-butyl ester, termed murabutide, non-toxic, non-pyrogenic adjuvant and inducer of non-specific resistance. Its capacity to suppress PFC formation in BALB/c mice is illustrated in Table VII.

TABLE VII

Suppression of PFC by Murabutide In Vivo

| Injection on* | Dose of MDP-BE (µg) | Mean No. of PFC** | % suppression |
|---|---|---|---|
| Day - 2 | 500 | 410 | 27 (p=.0002) |
| | 250 | 375 | 33 (p=.001) |
| | 125 | 482 | 14 (p=.001) |
| Day -1 | 500 | 415 | 26 (p=.002) |
| | 250 | 422 | 24 (p=.002) |
| | 125 | 455 | 19 (p=.03) |
| | 0 | 588 | |

*MDP-BE injected iv into BALB/c mice, 8 wk old, 1 or 2 days before $1 \times 10^8$ SRBC injected ip on day 0.

**Assayed on day +4; expressed as PFC/$2 \times 10^5$ spleen cells.

A comparison of its activity in 3 strains of mice is seen in Table VIII.

TABLE VIII

In Vivo Suppression of PFC by MDP-Butyl Ester in Different Strains of Mice

| Injected on* | PFC* | | |
|---|---|---|---|
| | Balb/C | C3H/HeN | C58 |
| Day -2 | 333 (30) | 155 (43) | 338 (34) |
| Day -1 | 278 (42) | 337 (0) | 320 (38) |
| Control | 478 | 273 | 515 |

*MDP-BE (500 µg) injected iv into 6 month old BALB mice 1 or 2 days before $1 \times 10^8$ SRBC injected ip on day 0. PFC assayed on day 5.
( )=percent suppression.

The second, a desmuramyl derivative L-ala-D-isogln-L-ala-$OCH_2CH(OH)CH_2O$-mycolate, termed triglymyc, also exhibited suppressive activity when given a day before antigen (Table IX).

TABLE IX

In Vivo Suppression of PFC by the MDP Derivative Muramyl tripeptide-glyceryl-mycolate*

| Injection ip on | Dose of TGM (μg) | Mean No. of PFC | % suppression |
|---|---|---|---|
| Day -2 | 500 | 106 | 54 (p = .003) |
| | 250 | 195 | 16 (p = .13) |
| | 125 | 333 | 0 |
| Day -1 | 500 | 106 | 55 (p = .002) |
| | 250 | 290 | 0 |
| | 125 | 377 | 0 |
| | 0 | 232 | |

Triglymyc injected ip into 8 wk old BALB/c mice 1 or 2 days prior to $1 \times 10^8$ SRBC given ip on day 0. PFC assayed on day +4.

The ability to transfer suppression with spleen cells by this desmuramyl derivative of MDP is documented in Table X. Thus, the muramyl group does not appear to be required for inducement of suppressor cells by the MDP class of adjuvants. The use of appropriate derivatives in future studies should aid in defining the relative importance of the different chemical entities in inducing suppression.

TABLE X

Transfer of Suppression with Triglymyc Treated Spleen Cells*

| Spleen Cells Transferred | PFC | | % suppression |
|---|---|---|---|
| | PBS | Tryglymyc | |
| TGM treated | 234 | 97 | 59 (p = .0000) |
| in vivo control: | 74 | 39 | 47 (p = .03) |

*Triglymyc injected ip on day -2 to Balb mice, spleens removed and transferred iv to recipient on day 0, SRBC injected ip and PPC measured on day 4.

## Non-specific Immunosuppression Induced by Polyribonucleotide Complexes

A second class of synthetic immunoadjuvants results from the complexing of opposite base pairs of the polyribonucleotides, ie. polyinosinic acid complexed with polycytidylic acid and polyadenylic acid complexed with polyuridylic acid. Such complexes act as adjuvants to both the helper and suppressor arms of the immune response to antigen depending on the time of their administration. The augmentation of suppression induced when such complexes are given to mice 1-2 days before antigen was documented in the initial annual report. Attempts to transfer to syngeneic BALB mice such suppression and identify the responsible cell were unsuccessful when varying numbers of spleen cells from poly A·poly U injected mice were injected into recipient mice at the same time as antigen, sheep red blood cells.

During the past year we have found that transfer of suppression was also unsuccessful when the cells were removed 6 hr or 15 hr as well as 24 hr after poly A·poly U injection and injected at the same time as antigen to syngeneic mice (Table XI).

Table XI

Failure to Transfer Suppression with Spleen Cells Removed Six or Fifteen Hours After Injection of Poly A·Poly U

| Cells Transferred ($10^7$) | Hours After poly A·poly U | Mean PFC |
|---|---|---|
| Control (PBS) | 15 | 103 ± 45 |
| Poly A·poly U | 15 | 170 ± 12 |
| Control (PBS) | 6 | 94 ± 50 |
| Poly A·poly U | 6 | 112 ± 31 |

In further experiments we switched from poly A·poly U to poly I·poly C, since the latter appeared to induce more formidable suppression when injected 1 day before antigen and consequently might more readily transfer this property. These transfer experiments revealed the day of transfer of the polynucleotide induced suppressor cells relative to antigen stimulation was all important. Thus, transfer of spleen cells removed from mice 1 day after being injected with poly I·poly C also was unsuccessful when given with antigen, but was successful in 4/6 mice given such cells 2 days after antigen. Transfer 3 days after antigen resulted in no suppression (and possibly enhancement) illustrating a precarious balance between suppressor and helper cell activity in polynucleotide exposed spleens. These preliminary experiments have been solidifed and repeated with poly A·poly U (Table XII).

Table XII

Transfer of Poly A·Poly U Induced Suppression

| Experiment* | PFC in recipient mice receiving cells from donor mice injected with | | |
|---|---|---|---|
| | PBS | A:U | % suppression |
| 1 | 468 | 286 | 39 |
| 2 | 505 | 166 | 67 |
| 3 | 141 | 65 | 54 |

*3 donor and 3 recipient mice/experiment. Mean difference in PFC (3 exp.) = 199 ± 76 (S.E.M.). Cells transferred 1 day after injection of poly A·poly U into syngeneic mice 2 days after latter received SRBC.

The duration of suppression induced in vivo by poly I·poly C also was studied by giving this suppressive agent on days -10, -8, -6, -4, -2, and -1 relative to antigen given on day 0. Definite suppression was found to last 6 days but was absent on day 8 and day 10 (Table XIII). It is of interest that this interval is similar to the duration of non-specific immunosuppression induced by MDP.

Table XIII

Duration of Suppression Induced by Poly I·Poly C

| Mice Injected With | PFC | | | | | |
|---|---|---|---|---|---|---|
| | Day of I·C injection Relative to Antigen | | | | | |
| | -1 | -2 | -4 | -6 | -8 | -10 |
| PBS (control) | 124 ± 10 | 333 ± 8 | 66 ± 15 | 279 ± 15 | 33 ± 17 | 262 ± 11 |
| Poly I·poly C | 44 ± 3 | 37 ± 1 | 11 ± 2 | 87 ± 5 | 200 ± 6 | 214 ± 21 |

Future experiments are designed to define the suppressive cell and whether its activity is mediated via secreted molecular entities.

## Characterization of Serum Suppressive Factors Induced by Polynucleotides

One of the facets of immunosuppression induced by the polynucleotides was the appearance in the serum 90 minutes after injection of factor(s) which suppressed the mixed leucocyte reaction (MLR). Suppression of the MLR (C58 x BALB) following the addition to the reaction well of murine sera removed 90 minutes after injection of each of 3 adjuvants is illustrated in Table XIV. Poly I·poly C-induced sera exerted the greatest suppression although poly A·poly U-induced sera also suppressed the MLR strongly. This suppression decreased significantly 18 hours after adjuvant injection in the case of poly A·poly U. Of interest was the finding that MDP-induced sera did not result in MLR suppression in 2 experiments. The suppressive effect of the sera on the MLR could not be diluted out to more than 1:10 in the case of poly I·poly C and to only 1:5 with two different poly A·poly U preparations. Consequently, fractionation and isolation of the factor(s) appears difficult.

Table XIV

Suppression of the MLR (C58 x BALB) by Adjuvant Induced Sera

| % suppression by 90 Minute Sera from Mice Injected with | | |
|---|---|---|
| Poly A·poly U | Poly I·poly C | MDP |
| 52 ± 19 (n=17) | 83 ± 5 (n=7) | 11 ± 0 (n=2) |

The decreased ability of aging animals to respond immunologically is well known. To test whether this might be due in part to the proficiency at which aged mice (or other immunodeficient models) might generate MLR-suppressive factors, blood was collected from 90-104 wk old BALB/c mice 90 minutes or 18 hours following poly A·poly U injection. Using young BALB/c spleen cells as responding cells no significant differences between sera from young and aging mice were seen in two experiments with respect to the suppressive 90 minute samples (34 vs 44% of control respectively). However, the suppressive effect disappeared more rapidly from the serum of aging as compared to young mice [i.e. the 18 hour poly A·poly U serum from young mice suppressed less (56% of control) than at 90 minutes, while poly A·poly U serum from aging mice at this time actually gave counts 26% higher than the control serum].

When the cells of aging BALB/c mice were used as targets the results were highly variable and cells from aging mice often gave severely defective MLR's. Consequently, it was difficult to draw conclusions regarding the effects of a suppressive serum; however, a similar trend appeared to exist as was seen using target cells from young mice, i.e. 90 minute poly A·poly U serum suppressed more strongly than 18 hour serum.

In order to rule out the possibility that the adjuvant-induced suppressive activity might be coming from the tissues since blood was collected from the axillary fossa, blood was also collected by cardiac puncture and the resulting sera tested in the MLR. A comparison of poly A·poly U and poly I·poly C sera resulting from both cardiac puncture and axillary bleeding showed suppression was maintained equally well by both serum samples from both sources.

To determine whether poly A·poly U had been retained in the sera and was responsible for the suppression, this adjuvant was added over a wide range of doses from .01 to 100 μg to the MLR and had virtually no effect.

Characteristics of the serum factor. Attempts to characterize putative suppressive factors present in the serum have been initiated. Results of 3 different experiments in which control serum and various adjuvant-induced sera were heated at 56°C for 45-60 minutes indicated that the MLR suppression appeared to be maintained after heating. Suppression also was maintained after freezing at -70°C for 1 month.

Since the heat stability of the suppressive factor(s) was compatible with that established for interferon, sera shown to be suppressive was tested for the presence of interferon. The results (Table XV) showed that the control serum had less than 10 units of interferon while the adjuvant-induced sera had significant interferon levels present. Marked differences were seen among 3 adjuvants in that the poly I·poly C-induced serum had 26,000 units of interferon while poly A·poly U-induced serum had only 110 units. Significantly, LPS also induced serum suppressive factors but with less interferon than poly I·poly C. The interferons present were also assayed as to type. In no case was gamma interferon measurable. Poly(A:U)-induced serum definitely had alpha interferon while the other adjuvant-induced sera were positive for beta interferon. Further studies are planned to clarify any relationships.

Table XV

Interferon Content of Adjuvant Induced Suppressive Sera

| Adjuvant Induced Serum | % Suppression | Interferon Units | Type of Interferon | | |
|---|---|---|---|---|---|
| | | | α | β | γ |
| Control | 0 | < 10 | - | - | - |
| poly A·poly U | 29 | 110 | ++ | ± | - |
| poly I·poly C | 85 | 26,000 | ± | ++ | - |
| LPS | 48 | 580 | ± | ++ | - |

The molecular size of the suppressive factor was also investigated utilizing the Amicon Centricon membrane system, with 30,000 m.w. and 10,000 m.w. cutoff membranes. In each of two experiments the suppression appeared in

the greater than 30,000 m.w. fraction with both poly A·poly U and poly I·poly C-induced sera. Data from one experiment appears in Table XVI. Similar results were achieved in the second experiment.

Table XVI

Molecular Weight Fractionation of Adjuvant-Induced MLR Suppressive Sera

| PBS serum fractions | % of MLR | AU serum fractions | % of MLR | IC serum fractions | % of MLR |
|---|---|---|---|---|---|
| unfractionated | 130 | unfractionated | 40* | unfractionated | 13* |
| > 30,000mw | 125 | > 30,000mw | 58* | > 30,000mw | 19* |
| 10,000-30,000 | 107 | 10,000-30,000 | 105 | 10,000-30,000 | 100 |
| < 10,000 | 121 | < 10,000 | 112 | < 10,000 | 106 |

*p = .00 as compared to PBS serum fractionation.

Background, Balb + Balb/m = 287 cpm (10% MLR)
MLR, Balb + C58/m = 12,690 (100% MLR)

Since soluble suppressive factors have been reported to be released from T cells, attempts were made to generate the MLR-suppressing factor in the serum of athymic nude mice. Poly A·poly U and poly I·poly C-induced sera each from 3 nude mice gave MLR suppression equivalent to that from normal mice (Table XVII). Thus, mature T cells may not be necessary for production and release of this suppressor factor.

Table XVII

Will Adjuvant-Induced Serum from Athymic Nude Mice Suppress the MLR

| PBS serum from | % of MLR | AU serum from | % of MLR | IC serum from | % of MLR |
|---|---|---|---|---|---|
| Balb/c | 154 | Balb/c | 93* | Balb/c | 34* |
| Athymic | 155** | Athymic | 86* | Athymic | 28* |
| Athymic | 119* | Athymic | 74* | Athymic | 27* |
| | | Athymic | 74* | Athymic | 16* |

*$p < .02$ as compared to PBS serum from Balb/c mice.

**$p = .98$ as compared to PBS serum from Balb/c mice.

Background, Balb + Balb/m = 1885 cpm (18% MLR).
MLR, Balb + C58/m = 10,425 cpm (100% MLR).

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